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Induction of 6-Thioguanine Resistance in Synchronized Human Fibroblast Cells Treated with Methyl Methanesulfonate, N-Acetoxy-2-Acetylaminofluorene and N-Methyl-N'-Nitro-N-Nitrosoguanidine

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16. ABSTRACT

Chemical induction of 6-thioguanine resistance was studied in synchronized human fibroblast cells. Cells initially grown in a medium lacking arginine and glutamine for 24 h ceased DNA synthesis and failed to enter the S phase. After introduction of complete medium, the cells progressed to the S phase after 16 h. DNA synthesis peaked 20 h after removal of nutrient stress and declined. Mutations were induced in S-phase cells by methyl methanesulfonate (MMS), N-acetoxy-2-acetylaminofluorene (NA-AAF) and N-methyl-N nitro-N-nitrosoguanidine (MNNG). Chemical treatments resulted in an increase in the absolute number of mutant colonies and in a dose-dependent mutation frequency. In this report, we show that NA-AAF evokes a temporal pattern of mutation in synchronized cells, with such mutations being induced only during the S phase. Evidence indicates that presence of S-phase cells in the treated cultures is a prerequisite for the induction of mutations.

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INDUCTION OF 6-THIOGUANINE RESISTANCE IN SYNCHRONIZED HUMAN FIBROBLAST CELLS TREATED WITH METHYL METHANESULFONATE, *N*-ACETOXY-2-ACETYLAMINOFLUORENE AND *N*-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE

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Summary

Chemical induction of 6-thioguanine resistance was studied in synchronized human fibroblast cells. Cells initially grown in a medium lacking arginine and glutamine for 24 h ceased DNA synthesis and failed to enter the S phase. After introduction of complete medium, the cells progressed to the S phase after 16 h. DNA synthesis peaked 20 h after removal of nutrient stress and declined.

Mutations were induced in S-phase cells by methyl methanesulfonate (MMS), *N*-acetoxy-2-acetylaminofluorene (NA-AAF) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Chemical treatments resulted in an increase in the absolute number of mutant colonies and in a dose-dependent mutation frequency. In this report, we show that NA-AAF evokes a temporal pattern of mutation in synchronized cells, with such mutations being induced only during the S phase. Evidence indicates that presence of S-phase cells in the treated cultures is a prerequisite for the induction of mutations.

Mutagens act primarily on replicating bacterial and viral DNA and preferentially mutagenize the replication point (Baker and Tessman, 1968; Cerda-Olmedo et al., 1968; Zampieri et al., 1968). Larger increases in the mutation frequencies for a particular gene occur when the mutagen is administered dur-

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Abbreviations: NMS, methyl methanesulfonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NA-AAF, *N*-acetoxy-2-acetylaminofluorene.

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ing the time in which the gene is being replicated (Cerda-Olmedo et al., 1968; Orkin and Littlefield, 1971; Burke and Fangman, 1975; Kee and Haber, 1975; Suzuki and Okada, 1975; Aebersold and Burki, 1976; Rasko et al., 1976; Tsutsui et al., 1978).

Studies (Taylor, 1960; Bader et al., 1963; Kikuchi and Sandberg, 1964; Mueller and Kajiwara, 1966; Mukherjee et al., 1968; Braun and Willi, 1969) indicate that DNA in the nucleus of mammalian cells replicates in a given chronology and pattern during the S phase, and experiments with synchronized cells have indicated that DNA synthesis is required for mutation. Mutants resistant to 6-thioguanine were induced only during early S phase by BUdR (Aebersold and Burki, 1976; Rasko et al., 1976; Tsutsui et al., 1978). The total percentage of cells synthesizing DNA paralleled the total DNA synthesis (Tsutsui et al., 1978; 1979). The occurrence of mutations appears to be related to the state of S phase, and the mutation frequencies to the number of S-phase cells in a mutagen-treated cell population.

The objective of this report is to provide information about conditions for inducing mutations in cultures of synchronized human fibroblasts. This report also describes the dose-dependent inductive 6-thioguanine (6TG) resistance in synchronized cells by different chemical agents, and the partial characterization of 6TG-resistant human fibroblasts.

Materials and methods

Cell lines, cultures media and cell cloning. Human fibroblast lines were derived from neonatal foreskins. Lesch—Nyhan cells (On Ser, $HGPRT^-$) were obtained from the American Type Culture Collection. Cells resistant to 6TG were derived from mutagenesis experiments. These cells were grown in 85 vol. of Ham's F10 medium (without hypoxanthine) and 15 vol. of fetal calf serum (FCS) with 100 units penicillin per ml and 0.1 mg kanomycin per ml at 37° in a 5% CO_2 atmosphere. 6TG-selective medium (FCS-F10-TG) consisted of FCS-F10 and 7.5×10^{-5} M 6TG. HAs medium consisted of FCS-F10, hypoxanthine (H) at 10^{-4} M and azaserine (As) at 2×10^{-6} M.

Cloning efficiency was measured by inoculating 100 cells into a 60-mm petri dish, renewing the medium after 1 week, and staining the cells at the 2nd week with 2% crystal violet after first rinsing with saline and fixing with 10% formaldehyde.

Determination of 6TG concentration that allows 6TG-resistant cells to grow among wild-type cells. 10 000 normal and 100 mutant cells, or 100 mutant cells alone, were plated in 60-mm dishes. The cultures were exposed to various concentrations of 6TG after 1 day. The selective medium was renewed once at the end of 1 week, and the cultures were stained for mutant colonies at the end of 2 weeks. The cloning efficiency of the mutant cells was determined concurrently in regular medium.

Cell synchronization by amino acid deprivation and determination of DNA synthesis. Human fibroblasts were synchronized by the method of Milo and DiPaolo, 1978. Logarithmically growing cells from preconfluent cultures were transferred to Dulbecco's modified Eagle's medium (MEM) (from Biolab), lacking arginine and glutamine and supplemented with 10% fetal calf serum, 1%

non-essential amino acid and 1% sodium pyruvate. After 24 h the cells were grown in MEM supplemented with 10% fetal calf serum plus insulin (0.5 IU/ml).

For determination of DNA synthesis, [^{3}H]thymidine was added to the above medium to a final concentration of 0.2 μCi/ml. The cells were labeled for 1 h, washed twice with saline, and finally dissolved in 0.1 N NaOH. At intervals of 1, 2, 3, 4, 5, 6, 7, 8, 16, 17, 18, 20 and 22 h following the release of nutrient stress, cultures received [^{3}H]thymidine and were labeled for 1 h. DNA in the lysate was precipitated with 5% cold tetrachloric acid and the lysate was filtered through a Whatman GF/C filter paper. The trapped DNA was washed twice with 5% cold TCA and then with 95% ethanol. The filter paper was dried and then transferred to a vial containing toluene-based scintillation fluid. The counts were obtained using a Packard Tri Car scintillation counter for each culture.

Induction of 6-thioguanine-resistant mutation in synchronized cell cultures during different periods of the G_1 and S phases. 1.5×10^6 cells were plated on 100-mm dishes, synchronized, and released from DNA synthesis blockage. At intervals of 7, 8, 16, 17, 18, 19, 20 and 22 h following the release of DNA synthesis blockage, the cultures were mutagenized by NA-AAF (4.2 μg/ml) for 1 h. After chemical treatment each dish of cells was washed twice with F10 medium. The cells were then cultured in FCS-F10 medium for 7 days with 2 passages. After 7-day expression, cells were plated in dishes, and the determination of mutation frequencies was done according to the method previously described (Jacobs and DeMars, 1977).

16-h mutagenesis with MMS in cells encompassing different cell phases. After release from DNA synthesis blockage, the synchronized cells in 100-mm culture dishes were treated with MMS for a period of 16 h. Treatment included the G_1 and S phases (8—24 h after release), S_1 and G_2 phases (16—32 h after release) and G_2 and G_1 phases (24—40 h after release). Mutation frequencies were estimated after 7-day expression following the method of Jacobs and DeMars (1978).

Mutagenesis by MNNG and NA-AAF. From 1.5 to 2 million cells were grown in medium deprived of amino acids for 24 h and then grown in complete MEM medium and insulin for another 16 h. These cells progressed into S phase and were exposed to the chemical for a period of 4 h. The treated cells were then cultured in FCS-F10 medium for 7 days with 2 passages.

For mutagenesis, 10^4 cells were plated per 60 mm dish. To determine the efficiency of mutant recovery, 100 6TG-resistant cells were added to this suspension (reconstruction dish), and 5 dishes were prepared for each dose. A group of 5 dishes containing only 100 6TG-resistant cells were also prepared for the determination of cell survival in the selective medium. All the dishes received 6TG at a final concentration of 7.5×10^{-5} M on the following day. The selective medium was renewed once every week. For determination of cloning efficiency, 100 cells per 60 mm dish were plated and grown in FCS-F10 medium. Reconstruction dishes and dishes for estimation of cell survival were stained at the end of the 2nd week and mutagenesis dishes at the end of the 3rd week. Mutant colonies were counted and the mutation frequencies were determined according to the Poisson expectation (Albertini and DeMars,

1973; Maher and Wessel, 1975; Jacobs and DeMars, 1977, 1978; Huang and Lieberman, 1978).

Characterization of spontaneous and induced mutants. Mutant colonies were isolated and grown in regular medium for 2 weeks before being stored in liquid nitrogen. Cells thawed from ampules were cultured for cell growth in different media. For a period of 1 week, 10^4 parental or mutant cells per dish were grown in groups in regular, 6TG, and HAs medium, respectively. Cells were then trypsinized and counted with a Coulter counter. The cell number per dish was determined.

Results

Optimum concentration of 6TG in selective medium. The present reconstruction experiments were performed to determine the maximum normal cell population density and/or the minimum concentration of 6TG at which mutant cells can form colonies at the same frequencies as when normal cells are also present. At higher non-mutant cell densities and/or low 6TG concentrations, the growth of normal cells obscures the recognition of mutant colonies.

Cells of different mutant lines could be recovered and formed colonies in medium containing 10^4 normal cells and 6TG concentrations that ranged from 2.5×10^{-5} M to 2×10^{-4} M (Fig. 1). The mutant recovery reached 80—100%

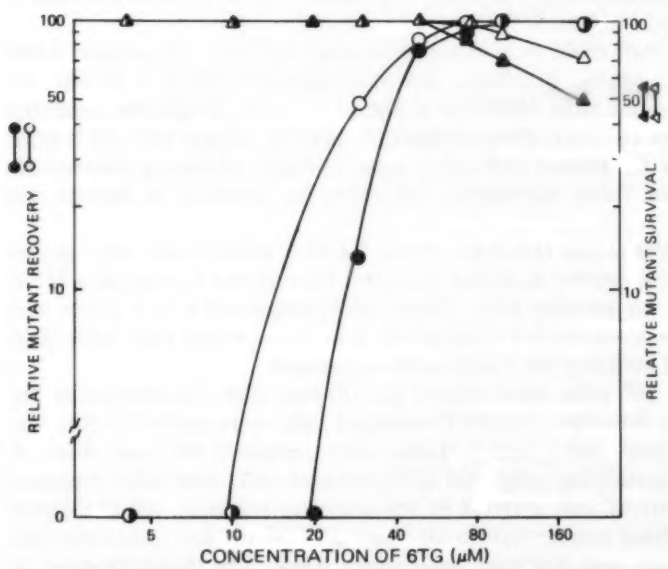

Fig. 1. The degree of mutant recovery and cell survival following 6TG exposure. 60-mm dishes containing mutant cells alone or together with 10^4 normal cells were exposed to different concentrations of 6TG and the medium was changed weekly. The dishes were stained after 2 weeks. Surviving mutant colonies were scored for each dish. At 7.5×10^{-5} M or higher, 6TG inhibited the growth of wild-type cells and allowed mutant cells to proliferate fully. At lower doses, 6TG allowed both mutant and wild-type cells to grow, and mutant colonies were not recognizable. 6TG was toxic to mutant cells and reduced their survival sharply at extremely high doses. Cell strains: On Ser (HGPRT$^-$, ●, ▲); induced mutant (○, △).

at 7.5×10^{-5} 6TG in different assays. Mutant recovery became poor with 6TG below 5×10^{-5} M and no mutant colonies were recognizable at 2×10^{-5} M or less. At doses higher than 7.5×10^{-5} M, where mutant recovery was estimated to be 100%, the survival of mutant cells decreased due to the cytotoxic effect of 6TG. As it was observed that 6TG at 7.5×10^{-5} M allowed good mutant recovery and survival, this concentration was employed in subsequent mutagenesis experiments.

Cell synchronization. Dulbecco's MEM lacking arginine and glutamine and supplemented with 10% fetal calf serum, 10% non-essential amino acid, and 1% sodium pyruvate stopped most cell growth, and rendered a concomitant decrease in DNA synthesis. Consequently the cells were blocked in G_1/S phase within 24 h. However, as shown in Fig. 2, some cells escaped blockage and continued synthesis of DNA. MEM supplemented with 10% fetal calf serum plus 0.5 IU insulin per ml released cells from G_1/S interphase blockage and new DNA synthesis began at 16 h and peaked 20 h after release. The increase in the DNA synthesis did not result from an increase in the cell number (Fig. 2).

Mutation induction was limited to different periods in S phase. 1-h expo-

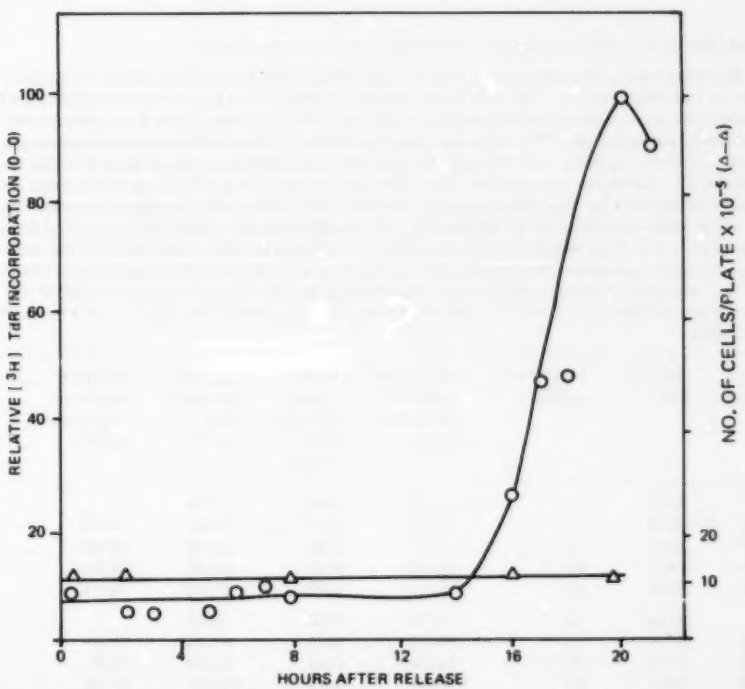

Fig. 2. Relative amount of DNA synthesis at different phases of cell cycle after release from blockage by the addition of complete medium. [^{3}H]TdR incorporation into the acid-insoluble fraction (○) and cell number (△) were measured after release. Cells progressed to S phase 16 h after the release and DNA synthesis peaked after 20 h. Increase in the amount of DNA synthesis was not due to an increase in the cell number, where the cell number per plate stayed the same for 20 h after introduction of complete medium.

TABLE 1

MUTATION FREQUENCIES INDUCED BY NA-AAF DURING DIFFERENT PERIODS IN THE G_1 AND S PHASES

h after release	cpm [a]	Number of cells/dish ($\times 10^{-5}$)	Mutation [b] frequency ($\times 10^6$)	
			Control	Treated
7	263	1.31	3.9	0
8	215			0
16	804	1.44	11	29
17	1427	1.39		
18	1448			96
20	3065	1.39		127
22	2600		10	76

[a] Cells were labeled with [^{3}H]thymidine at 0.2 μCi/ml in complete medium for 1 h. Counts per min are based on the total cell population per 60-mm dish. DNA synthesis peaked 20 h after release of DNA synthesis blockage.

[b] Mutagen treatment lasted for 1 h. Determination of mutation frequencies was performed according to Jacobs and DeMars (1977).

TABLE 2

MUTATION INDUCTION BY MMS IN HUMAN DIPLOID FIBROBLAST CELLS

Human foreskin fibroblasts were synchronized in arginine—glutamine-deficient medium for 24 h. Approx. 16 h after change to complete medium, the cells were exposed to MMS for 16 h encompassing different cell phases. Treated cells were cultured in F10 medium with 10% FCS for 7 days with 2 subcultures during this period. For mutant selection 10^4 cells were plated per 60 mm dish and 100 cells were plated per dish for estimation of survival; cultures were fed weekly. Cultures were fixed and stained after 2 weeks for survival and 3 weeks for estimation of mutants. The corrected number of mutants was determined by dividing the number of mutants by the relative mutant recovery (from reconstruction experiments). The total number of viable cells was obtained by multiplying the number of cells plated by the survival fraction. Mutation frequency was then determined by dividing the corrected number of mutants by the number of viable cells. Induced mutation frequencies were obtained by subtracting the background mutation frequency from the calculated mutation frequency. The straight line fit for the induced mutation frequencies over the doses are $\ln(y) = -0.653 + 1.339 \ln(\lambda)$ for $G_1 + S$ phases, and $\ln(y) = -0.49 + 0.98 \ln(x)$ for $S + G_2$ phases.

Cell phase	Concn. (μg/ml)	Survival (%)	Number of mutants	Corrected number of mutant clones	Total number of viable cells ($\times 10^5$)	Mutants per viable cells ($\times 10^6$)	Induced mutation frequency ($\times 10^6$)
$G_1 + S$	0	32.4	1	1.91	2.66	7.18	
	6.25	32.6	2	3.52	2.97	11.86	4.68
	12.50	28.8	4	6.47	2.53	25.58	18.40
	25.00	28.2	10	14.66	2.43	60.34	53.16
	50.00	22.8	10	16.18	1.98	81.72	74.54
$S + G_2$	0	46.8	2	6.73	3.70	18.19	
	6.25	47.0	4	15.74	3.85	40.88	21.81
	12.50	47.4	6	23.62	3.93	60.10	41.91
	25.00	44.0	10	40.55	3.83	105.87	87.68
$G_2 + G_1$	0	21.6	3	5.08	2.38	21.36	
	3.13	19.0	3	5.74	2.09	27.44	
	6.25	20.2	0	0	2.22	0	
	12.50	12.4	0	0	1.36	0	
	25.00	27.6	0	0	3.04	0	

sures of cells to NA-AAF at different stages of the cell cycle allowed observation of the responsiveness of cells to mutagen damage. Exposure of cells in the G_1 phase produced no obvious mutations, as demonstrated in Table 1. However, exposure of mutagens to cells synthesizing DNA induced mutations. The mutation frequencies increased as the S phase progressed and were the highest when the DNA synthesis reached a maximum. Then the mutation frequencies declined as the S phase passed the mid-point even though DNA synthesis continued in some portion of the cell population.

Mutation induction by MMS. The treatment, starting from G_1 and including the entire S phase, induced mutations in a dose-dependent fashion. The treatment that included S phase and G_2 phase also induced mutations in a dose-dependent fashion. However, the treatment that included G_2 and G_1 did not induce mutations (Table 2). Induction of 6TG resistance was linear with increasing doses and mutation induction followed one-hit kinetics. Interestingly both slopes of regression appear identical. These findings indicate that incorporation of mutagens into synthesizing DNA is required for mutation.

Mutagenesis with MNNG and NA-AAF. The estimation of mutation frequencies was based on the exact probability function for the Poisson random vari-

TABLE 3

CALCULATION OF INDUCED MUTATION FREQUENCIES AFTER TREATMENT WITH NA-AAF

The distribution of mutant colonies per dish was tabulated. The average number of mutant colonies (X) per dish was obtained according to $P_0 = e^{-X}$, where P_0 was the fraction of dishes bearing no mutants. The negative log of P_0($-\ln P_0$) gave a mean number of mutants per dish (X). Mutant cells do not form colonies when grown with too many normal cells. Their plating efficiency decreases as the number of normal cells increases. To correct for underestimation of X due to the inhibition of clone formation by wild-type cells, X values were divided by the respective mutant-recovery value (estimated from reconstruction experiments), giving a "corrected X". Multiplication of the initial number of cells per dish by the cloning efficiency of the cells gave the number of surviving cells per dish. Mutation frequencies were calculated by dividing "corrected X" by the number of surviving cells per dish. Induced mutation frequencies were obtained by subtracting the background mutation frequency from the calculated mutation frequencies

Dose of NA-AAF (μM)	0	7.5	10	12.5	17.5
Dishes scored (number)	105	108	107	110	110
Dishes with 0 clones	103	102	100	101	95
Dishes with 1 clone	2	6	7	9	10
Dishes with 2 clones	0	0	0	0	2
Dishes with 3 clones	0	0	0	0	3
Total mutant clones	2	6	7	9	23
$P_0 = \frac{\text{Dishes without clones}}{\text{Total dishes scored}}$	0.951	0.944	0.935	0.918	0.864
X = average number of mutant clones per dish	0.019	0.057	0.068	0.085	0.147
Efficiency of mutant recovery	0.84	0.87	0.94	0.92	0.94
Corrected X	0.023	0.066	0.072	0.092	0.156
Initial cell number/dish	12 000	9360	12 000	10 560	11 880
Cloning efficiency	0.315	0.236	0.108	0.055	0.042
Number surviving cells/dish	3 780	2208	1 296	575	504
Mutation frequency ($\times 10^6$)	6.05	29.78	55.57	161.00	309.4
Induced mutation frequency ($\times 10^5$)	0	2.37	4.95	15.50	30.33

able (Table 3). In the present studies, 4-h treatment of S-phase human fibroblasts with NA-AAF and MNNG induced mutation. Data from these experiments are plotted in Figs. 3 and 4. Production of 6TG-resistant clones was linear with increasing concentration of chemicals (in log transformation). Induction of mutation by MNNG follows one-hit kinetics. The mutation resulting from NA-AAF treatment followed higher-order function ($b = 3.31 \pm 0.28$).

Characterization of mutants. 100 mutant lines were evaluated for growth in 6TG-containing medium and HAs medium. All 100 lines showed 6TG resistance. Of these mutant lines 16% were able to use exogenous hypoxanthine for growth. These probably correspond to the type-II mutants (DeMars, 1974). The mutants derived from synchronized cells resemble Lesch—Nyhan cells and other 6TG- and 8AG-resistant mutants (Albertini and DeMars, 1973; Van Zeeland et al., 1974; Huang and Lieberman, 1978; Jacobs and DeMars, 1978).

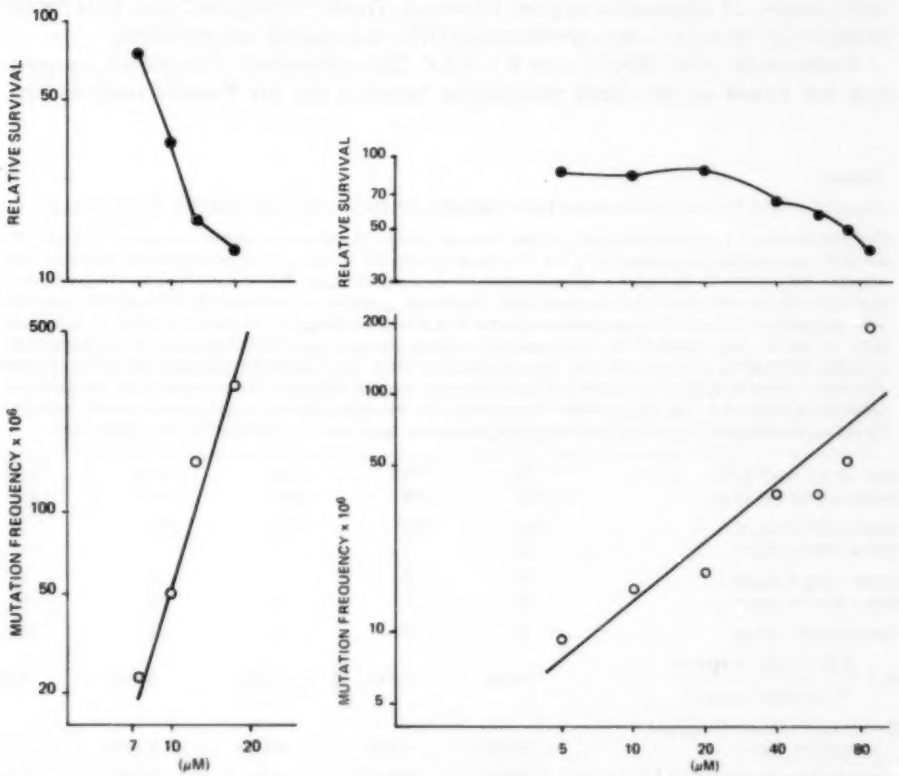

Fig. 3. Mutagenicity and cytotoxicity in synchronized human fibroblasts treated with NA-AAF for 4 h. After a 7-day expression, the cytotoxic effect of NA-AAF continued. The straight line fit for the induced mutation frequencies over the doses tested is given by: $\ln(y) = -5.428 + 3.127 \ln(x)$. Detailed data are presented in Table 3.

Fig. 4. Mutagenicity and cytotoxicity in synchronized human fibroblasts treated with MNNG for 4 h. The cytotoxic effect of MNNG in treated cells continued after a 7-day expression. Induction of mutation was dose-dependent. The fitted straight line is given by: $\ln(y) = 0.793 + 0.800 \ln(x)$.

Discussion

This method of cell synchronization by amino acid deprivation (Milo and DiPaolo, 1978) proved useful for obtaining synchronized human fibroblast cultures. New DNA synthesis began at 16 h and peaked 20 h after release from nutrient stress. This permitted a division of the S phase into 8 1-h periods. Experiments with synchronized cells allow one to determine whether DNA synthesis is required for the mutation of cells in cultures induced by mutagen treatment. The cellular effects of a direct perturbation of DNA during various portions of the DNA synthetic period (S) phase were examined. The incidence of mutation was dependent on the portion of the S phase during which treatment was administered. Similar results have been reported at the HGPRT locus in Chinese hamster (Aebersold and Burki, 1976; Rasko et al., 1976) and Syrian hamster cells (Tsutsui et al., 1978). This report and others show that only S-phase cells were sensitive to mutagens, and inclusions of S-phase cells was necessary for mutagenesis experiments (Taylor, 1960; Bader et al., 1963; Kikuchi and Sandberg, 1964; Mueller and Kajiwara, 1966; Mukherjee et al., 1968; Braun and Willi, 1969).

Mutation can be induced in logarithmically growing human cells (Albertini and DeMars, 1973; Cox and Masson, 1974; Van Zeeland et al., 1974; Kuroda, 1975; Maher and Wessel, 1975; Jacobs and DeMars, 1978; Huang and Lieberman, 1978). These logarithmically growing cultures contain various proportions of S-phase cells in their populations. Cell culture synchrony was used here to accumulate larger numbers of S-phase cells for mutagen treatment and to increase the sensitivity of the testing system.

In the present studies, treatment of S-phase human fibroblasts with MMS, NA-AAF and MNNG induced mutation. Production of TG-resistant clones was linear with increasing concentration of chemicals, and, with the exception of NA-AAF, appeared to follow one-hit kinetics. The mutation resulting from NA-AAF treatment followed a higher order function ($b = 3.31 \pm 0.28$). NA-AAF and MNNG were tested and were shown to be mutagenic to human foreskin fibroblasts with 3- and 5-day expression times, respectively (Maher and Wessel, 1975; Huang and Lieberman, 1978; Jacobs and DeMars, 1978). Use of cells in synchrony appeared to be more sensitive than the methods previously used (Maher and Wessel, 1975; Huang and Lieberman, 1978). However, Jacobs and DeMars (1978) could induce more mutations with MNNG in logarithmically growing cultures which yield a larger slope than that reported in this paper. With human cells in synchrony all three chemicals resulted in increased mutation frequencies as well as an increased absolute number of mutant colonies.

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